Linker-Insertion Nonsense and Restriction-Site Deletion Mutations of the gB Glycoprotein Gene of Herpes Simplex Virus Type 1

WEIZHONG CAI,¹ STANLEY PERSON,^{1*} SUSAN C. WARNER,¹ JIANHUI ZHOU,¹ AND NEAL A. DeLUCA²

Department of Molecular and Cell Biology, The Pennsylvania State University, University Park, Pennsylvania 16802,¹ and Dana-Farber Cancer Institute, Harvard University, Boston, Massachusetts 02115²

Received 22 July 1986/Accepted 31 October 1986

To study the effects of missense, nonsense, and deletion mutations of the gB glycoprotein gene of herpes simplex virus type 1, a gB-transformed cell line was isolated that, after virus infection, would express sufficient quantities of gB from the cellular chromosome to complement temperature-sensitive gB mutants. The transformed cell line was then used as a permissive cell to transfer two gB mutations from plasmid to viral DNA. One of the mutants, K082, harbored an *HpaI* linker insertion that introduced one new amino acid and a chain terminator codon within amino acid residue 43. The other mutant contained a 969-base-pair deletion in a part of the gene that includes the membrane-spanning region; a correspondingly shorter gB polypeptide was detected by sodium dodecyl sulfate-gel electrophoresis after immunoprecipitation of infected-cell extracts with four pooled monoclonal antibodies. No polypeptide was observed from K082-infected cells. The shortened gB polypeptide was efficiently processed and secreted into the growth medium. Each of the four monoclonal antibodies precipitated full-length gB, and three of the four precipitated the shortened polypeptide. Enveloped virus particles could be purified after infection of nonpermissive cells with either mutant virus. Virus particles appeared to possess normal polypeptide and glycoprotein profiles except for the absence of gB. Therefore, the presence of gB is not essential for viral assembly, including envelopment. Recombinants in virus stocks grown on the gB-transformed cells occurred at frequencies on the order of 10^{-7} to 10^{-5} , compared with a frequency of approximately 10^{-2} in mixed infections with the two mutants.

Glycoproteins on the surfaces of viruses are important for viral adsorption and entry into host cells. For herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) several pairs of homologous glycoproteins, designated gB, gC, gD, and gE, are encoded by the viral genomes and expressed on the virion surface (for a review, see reference 40). Two other glycoproteins have been identified, one in HSV-1, gH-1 (4), and another in HSV-2, gC-2 (33). (Accepted nomenclature includes the use of a suffix, -1 or -2, to indicate a glycoprotein specified by HSV-1 or HSV-2, respectively. Here, for simplicity, gB will be used to indicate gB-1). An HSV-1 glycoprotein that may be the HSV-1 counterpart of gC-2 was recently identified by Richman et al. (32). Although glycoproteins must be important to virus function in vivo, little is known about their role in virus growth. Infectious virions are formed in vitro in the absence of gC-1 (18, 24) or gE-1 (23). Glycoprotein gB is the only glycoprotein currently known to be essential for viral growth as revealed by experiments with mutants carrying temperature-sensitive (*ts*) mutations in the gB gene (24, 36, 37). The gB glycoprotein has a role in infectious entry (10, 22, 35) and in cell fusion (6, 24).

The DNA sequence of the gB gene has been reported for two strains of HSV-1 (5, 29). The ATG initiation codon is at 0.366 and the TGA termination codon is at 0.348 prototypic map units (2.7 kilobase pairs [kb]). The gB polypeptide has characteristics typical of a transmembrane protein. Secondary structure predicts three passages of a hydrophobic region of the protein through the membrane (29). A relatively long C terminus (109 amino acids) protrudes into the cytoplasm, which may imply a function for this region of gB. A single amino acid difference between the *tsB5* and KOS

strains causes extensive fusion of Vero cells (6; W. Cai, M.S. thesis, The Pennsylvania State University, University Park, 1985). A single amino acid change in the external portion of gB causes a change in the rate of viral entry between KOS and other strains (6, 10).

Clarification of gB function has been aided by the existence of three types of gB mutations—temperature sensitivity for viral growth (12, 24, 36, 37), resistance to virus neutralization by monoclonal antibodies (19, 20), and the syncytial plaque morphology of *tsB5* (24). To determine the role of various domains of gB on its synthesis, processing, and transport, on virus assembly, and on virus penetration into host cells, mutants are needed that include nonsense and deletion mutations. A second requirement is for a gB-transformed cell line providing gB in *trans* to complement these defects in the viral genome. This strategy has been used successfully for the isolation of the mutants of polyoma virus (2), simian virus 40 (SV40) (13), adenovirus (17), and Moloney murine leukemia virus (7). There have been several reports on the use of HSV-1 genes to transform mammalian cells (1, 9, 11, 28, 34, 44), but only DeLuca et al. (11) have used transformed cells to construct viral mutants defective in an essential viral gene. In this paper we report the development of such a cell line. We also report here the construction, isolation, and partial characterization of two viruses, one containing a nonsense and the other a deletion mutation within gB coding sequences.

(A preliminary account of this work was given at the Tenth International Herpesvirus Workshop, University of Michigan, 1985.)

MATERIALS AND METHODS

Cells and viruses. Human embryonic lung (HEL) cells were grown and maintained as described by Person et al.

* Corresponding author.

(31). Vero cells purchased from Flow Laboratories, Inc., and gB-transformed Vero cells, designated D6 (see below), were grown and maintained as for HEL cells except that for routine harvesting a 0.03% trypsin–2 mM EDTA solution in a buffered salt solution (TBS) (31) was used, whereas 0.02% trypsin–2 mM EDTA was used for HEL cells. The growth medium for D6 cells included 200 μ g of G418 (Geneticin; Gibco Laboratories) per ml during the first passage of cells from the freezer.

Escherichia coli RDP145 (*galB2 sbcB15 hsdR4 endA* Nal^r Tr^r *recA13*), constructed and provided by Ronald D. Porter (this department), was used for the isolation and amplification of plasmid DNA. The *E. coli* strain was grown in VG medium (10 g of tryptone, 5 g of yeast extract, 7 g of NaCl, 0.5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 2 g of glucose in 1 liter of water) or VG plates (VG medium plus 15 g of agar per liter). Ampicillin (100 μ g/ml) was added to select appropriate ampicillin-resistant plasmids.

HSV-1 strains KOS and *tsJ12* (36) were obtained from Priscilla Schaffer (Harvard Medical School, Cambridge, Mass.), and *tsJ20* was isolated in this laboratory (12). Viruses were grown and maintained as described by Person et al. (31). Virus stocks of the gB-defective mutant strains KAT and K082 were grown on D6 cells.

Plasmids. pKBXX was constructed by inserting a 4-kb HSV-1 *Bam*HI–*Xho*I fragment (0.345 to 0.372) from pKBG-BS3 (10) into *Bam*HI–*Sal*I sites in pUC9 (42). This fragment contains the gB coding region (see Fig. 2). Partial digestion with *Xho*I was necessary because of a second *Xho*I site near 0.370 (nucleotides 538 to 543). The desired 4.0-kb fragment was identified by restriction mapping. pKAT was constructed by deleting the *Bst*EII fragment from pKBXX. pK082 has a hexanucleotide *Hpa*I linker (GTTAAC) inserted in the HSV-1 sequence of pKBXX about 3 kb from the *Bam*HI site. It is part of a collection of plasmids with an *Hpa*I linker inserted into one of a large number of *Alu*I, *Fnu*DII, *Hae*III, and *Rsa*I restriction sites in pKBXX. The construction, isolation, and mapping of these plasmids will be presented elsewhere. pSP65-BXX was constructed by inserting the 4-kb HSV-1 *Bam*HI–*Hind*III fragment in pKBXX into the *Bam*HI and *Hind*III sites in pSP65 (27), obtained from Promega Biotech (Madison, Wis.). pSV2-neo contains the G418 resistance marker under control of the SV40 early promoter (39).

DNA isolation. HSV-1 DNA was isolated by the procedure of Goldin et al. (13). Small batches of plasmid DNA were isolated by the procedure of Birnboim and Doly (3); large batches were further purified by equilibrium sedimentation in CsCl . Mammalian cellular DNA was isolated by the method of Stow et al. (41).

Transformation of Vero cells. The procedure of DeLuca et al. (11) was followed for transformation of Vero cells. Subconfluent Vero cells (2×10^6) were seeded in a 100-mm-diameter petri dish and incubated overnight. Cells were cotransformed with calcium phosphate-precipitated DNA (1.2 μ g of pKBXX, 0.4 μ g of pSV2-neo, and 20 μ g of salmon testes carrier DNA [Sigma Chemical Co.] [16]) followed by 15% glycerol shock 4 h later. G418 was then added at 1.0 mg/ml, and the concentration was lowered to 500 μ g/ml 10 days later. Individual colonies were isolated and tested for their ability to support the growth of gB *ts* mutants. One of the cell lines, designated D6, complemented the *ts* mutation significantly and was chosen for further characterization and other studies.

Marker rescue and marker transfer. Marker rescue and transfer of mammalian cells with intact HSV-1 genomic

DNA and a cloned DNA fragment were done essentially as described by Graham and Van der Eb (17). Cells (4×10^5) in 5 ml of growth medium were seeded in a 60-mm-diameter petri dish 24 h before transfection and incubated at 37°C. The medium was changed 3 h before transfection. Approximately 1 μ g of HSV-1 genomic DNA, 1 μ g of restriction-digested, cloned DNA, and 10 μ g of salmon testes carrier DNA in 250 μ l of transfection buffer (10) were mixed with an equal volume of 250 mM CaCl_2 for 20 min to precipitate the DNAs. The sample was then added to the petri dish containing cells and incubated at 37°C for 4 h. The DNA– Ca^{2+} -treated cells were shocked by 15% glycerol in TBS for 2 min. After the cells were washed, 5 ml of fresh growth medium was added, and the culture was incubated for 5 to 7 days at 37°C until a cytopathic effect was observed for essentially all of the cells.

Southern blot hybridization. Restriction-digested DNA fragments were electrophoresed on an agarose gel and transferred onto a nitrocellulose filter (38). Southern hybridization was conducted by the method of Melton et al. (27). A [32 P]GTP-labeled RNA probe was synthesized from *Bam*HI-digested pSP65-BXX by sp6 RNA polymerase. The nitrocellulose filter was prehybridized in hybridization buffer (50% formamide, 6 $\times$ SSC [1 $\times$ SSC is 0.15 M NaCl, 0.015 M sodium citrate], sodium dodecyl sulfate [SDS], 5 $\times$ Denhardt solution, and 200 μ g of denatured salmon testes DNA per ml) and hybridized overnight in the presence of probe (5 $\times$ 10⁶ dpm). The filter was washed twice for 30 min each at room temperature in 1 $\times$ SSC containing 0.1% SDS and then washed twice for 30 min each at 65°C in 0.1 $\times$ SSC containing 0.1% SDS. Labeled, unhybridized RNA was reduced by RNase A treatment. The filter was rinsed three times each for 5 min in 2 $\times$ SSC, incubated for 15 min at room temperature with RNase A (1 μ g/ml) in 2 $\times$ SSC, and washed for 30 min at 50°C in 0.1 $\times$ SSC containing 0.1% SDS.

Virion purification. Vero cells (10^8) in roller bottles were infected with viruses at a multiplicity of infection (MOI) of 10 PFU/cell and labeled from 6 to 24 h postinfection. The cultures were scraped into the medium, pelleted, and suspended in TBS. After three freeze-thaw cycles, the cell debris was pelleted and discarded. The virus in the supernatant was pelleted for 30 min in a microfuge and suspended in 100 to 200 μ l of TBS. The samples were loaded onto Dextran T10 gradients (1 to 30%) for centrifugation. The collected fractions were assayed for radioactivity by liquid scintillation counting. Peak fractions were further loaded in sucrose (35 to 65%) gradients for centrifugation, if necessary.

Immunoprecipitation. The procedure described by Holland et al. (19) was used for immunoprecipitation. Briefly, virus-infected cells were lysed in Nonidet P-40-containing lysis buffer. The extracellular medium was collected separately. Growth medium was added to the Nonidet P-40 cellular extract to the same volume as extracellular medium. A few microliters of pooled monoclonal gB antibodies (B3, B4, B6, and B9, kindly provided by J. Glorioso, University of Michigan, Ann Arbor) was added and incubated at room temperature for 3 h or at 4°C overnight. After incubation with protein A-Sepharose beads (Pharmacia, CL-4B) for 1 h, the beads and bound antigen-antibody complex were pelleted and washed three times in lysis buffer. Samples were suspended and heated for 5 min to release the antigen, and proteins were separated by SDS-polyacrylamide gel electrophoresis by the method of Laemmli (21) as modified by Manservigi et al. (24).

Materials. [32 P]GTP was obtained from New England Nuclear Corp. Restriction enzymes and T4 DNA ligase were purchased from Bethesda Research Laboratories and New

TABLE 1. Complementation of gB mutants by D6 cells^a

Virus	Growth (PFU/ml)			
	D6		Vero	
	34°C	39°C	34°C	39°C
KOS	2.0×10^{10}	1.6×10^{10}	1.3×10^{10}	1.2×10^{10}
<i>tsJ12</i>	6.6×10^9	5.6×10^9	4.8×10^9	3.3×10^6
<i>tsJ20</i>	6.2×10^9	8.1×10^9	4.2×10^9	$<1 \times 10^5$

^a Monolayers of D6 and Vero cells were infected with the same concentration of each virus and incubated at 34 or 39°C. Plaque number was determined 3.5 days later.

England BioLabs. The enzymes used in preparation of RNA probes, SP6 polymerase and DNase I, were obtained from Promega Biotec. Reactions with these enzymes were conducted as specified by the manufacturers.

RESULTS

Isolation of a gB-transformed cell line. The gB gene of HSV-1 is essential for viral replication. To isolate gB-defective mutants, a cell line that expresses gB is required that will support the growth of these mutants. Therefore, Vero cells were cotransfected with plasmids pSV2-neo, which specifies the G418 resistance gene, and pKBXX, which contains the gB coding region (and approximately 800 and 500 base pairs [bp] of 5'- and 3'-flanking sequences, respectively) of the KOS strain of HSV-1. After growth in the presence of G418 for 18 days, 24 colonies were tested qualitatively for the ability to support the growth of the gB *ts* mutants *tsJ12* and *tsJ20* at the nonpermissive temperature. Plaques were observed repeatedly for six of the transformed cell lines. One of these, designated D6, yielded the highest level of complementation. Vero cells were also transformed with p52BXX, which contains the gB-2 gene of the HG52 strain of HSV-2, and pSV2-gB, which has the gB coding region of KOS under control of the SV40 early promoter. Since none of these G418-resistant colonies showed a greater complementation ability than D6, D6 was chosen for further study.

To further test the plaque-forming efficiency of D6, monolayers of both D6 and Vero cells were infected with *tsJ12*, *tsJ20*, or KOS virus and incubated at 34 or 39°C (Table 1). The wild-type KOS formed plaques on Vero cells almost as well as on D6 at both temperatures. Although *tsJ12* and *tsJ20* formed plaques efficiently on both cells at 34°C, they formed plaques at the wild-type level at 39°C only on D6. Titers were higher on D6 than on Vero cells by several orders of magnitude. This result demonstrated that D6 could provide functional gB for the *ts* mutants at the nonpermissive temperature. D6 was routinely grown in the laboratory for up to 12 passages in the absence of G418 without the loss of complementation ability.

To confirm that D6 contained the gB gene, Southern blot analysis was performed to detect gB-specific DNA sequences. DNA was isolated from D6 and Vero cells and digested with the restriction enzymes *Bam*HI and *Hind*III. The DNA fragments transferred to a nitrocellulose filter after agarose gel electrophoresis were hybridized with a ³²P-labeled RNA probe whose sequences were the same as the *Bam*HI-*Hind*III fragment in pKBXX. The expected HSV-1 fragment was detected in D6 and was present in approximately 10 to 20 copies per cell (Fig. 1).

Isolation of gB-defective mutant viruses. gB mutant plasmids were derived from pKBXX (Fig. 2). pKΔT was con-

structed by *Bst*EII digestion and religation to delete the 969-bp *Bst*EII fragment. The deletion includes the gB gene membrane-spanning region and one of the potential N-linked glycosylation sites predicted from the DNA sequence (Asn-Ile-Thr at residues 674 to 676). pK082 has a 6-bp *Hpa*I linker inserted into pKBXX. There are no *Hpa*I sites in the HSV-1 insert or in the pUC9 vector. pKBXX was partially digested with a dilute solution of *Alu*I, *Fnu*DII, *Hae*III, and *Rsa*I. Full-length linearized DNA was isolated and ligated with *Hpa*I linkers. pK082 is one of 82 plasmids identified after transformation of *E. coli* mapping data (Fig. 2), and reference to DNA sequence analyses (5; D. J. Bzik et al., in press) showed that the *Hpa*I linker (GTTAAC) was inserted after the first nucleotide of the codon for amino acid 43 of the gB gene, disrupting this codon (GCG) for alanine and creating three new codons, GGT, TAA, and CCG. Glycine (GGT) is inserted in place of alanine in codon 43, and polypeptide chain termination occurs at the site of the adjacent TAA codon.

The mutations in pKΔT and pK082 were transferred into the viral genome by marker transfer in which D6 cells were cotransfected with each of the plasmids and the intact viral DNA. pK082 was used in cotransfection experiments with KOS genomic DNA. Two hundred plaques resulting from the cotransfection culture were tested for growth on D6 and Vero cells. Two of the viruses could grow on D6 but not on Vero cells. They were the same in terms of *Hpa*I restriction pattern in Southern blots. Therefore, one of them, designated K082, was chosen for further analysis. The mutation in pKΔT was transferred to viral DNA by a similar strategy to generate a virus designated KΔT.

The following experiments were performed to confirm that the defects in K082 and KΔT were caused by the gB mutations present in pK082 and pKΔT. First, small batches of viral DNA were isolated, digested with appropriate restriction enzymes, and subjected to Southern blot analysis (Fig. 3). The 8.2-kb *Bam*HI G fragment of KOS DNA was divided into 5.1- and 3.1-kb fragments by simultaneous digestion of K082 DNA with *Bam*HI and *Hpa*I. Comparison of *Bst*EII digests of KOS and KΔT DNA revealed the absence of a 1-kb fragment from KΔT DNA. The locations of these alterations were the same as those in the plasmids used

FIG. 1. Detection of HSV-1 DNA in D6 cells. DNA samples (10-μg) isolated from D6 and Vero cells were digested with *Bam*HI and *Hind*III and electrophoresed on an 0.75% agarose gel. Different amounts of pKBXX (20, 100, and 200 pg) were also added to Vero DNA in restriction reactions to indicate the number of copies (1, 5, or 10, respectively) of viral DNA per diploid cell. DNA fragments were then transferred onto a nitrocellulose filter and hybridized with a ³²P-labeled RNA probe prepared from the *Bam*HI-*Hind*III fragment in pSP65-BXX.

FIG. 2. Mapping of gB plasmid mutations in plasmids. (a) Restriction maps of pKBXX, pKΔT, pK082, and pΔ082/030 cloned into pUC9. Abbreviations: B, *Bam*HI; E, *Eag*I; H, *Hind*III; Hp, *Hpa*I; N, *Not*I; T, *Bst*EII. The gB coding region which specifies 904 amino acids is included, starting at ATG (right) and ending at TGA (left). The signal sequence (right) and membrane-spanning domain (left) are represented by black boxes. The *Hind*III site is present in the multicloning site of the vector. Nucleotide 1 of the HSV-1 insert is at the *Sall-Xho*I hybrid site immediately to the left of the *Hind*III site. The *Bst*EII sites (GGTCACC) begin at the third nucleotide of codons 552 and 875. Cleavage with *Bst*EII occurs between codons 552 and 553 and between 875 and 876. The coding strand is deleted from codon 553 through codon 875. Ligation joins codon 552 to 876 and produces a 323-amino-acid deletion without changing the reading frame. (b) Map of the *Hpa*I insertion in pK082. The nearby restriction sites relevant to mapping are included, as are the locations of amino acid residues 41 to 43. The position of the *Hpa*I insertion was determined initially by restriction mapping with *Bam*HI-*Hpa*I and *Hind*III-*Hpa*I digestion. The new *Hpa*I site in pK082 was mapped 3.1 kb from the *Bam*HI site, or 0.9 kb from the *Hind*III site. Further mapping revealed that the *Hpa*I insertion destroyed the unique *Not*I site at nucleotides 911 to 918. An *Hae*III site (GGCC) and two *Fnu*DII (CGCG) sites are involved in the *Not*I site, so the insertion is at one of these sites. *Not*I recognizes 8 bases GCGGCCGC, whose central hexanucleotide CCGCCG is recognized by *Eag*I, a site that was not destroyed by linker insertion. This eliminated the *Hae*III site at the center of the *Eag*I site as the point of insertion. Deletion of a fragment in the 3' direction from the *Hpa*I site in pK082 to generate plasmid pΔ082/030 (to be described elsewhere) did not eliminate the *Eag*I site. This indicated that the *Hpa*I linker was inserted at the 3' *Fnu*DII site. The arrow indicates the site of the *Hpa*I linker insertion. In panel b the direction of transcription is left to right.

to generate the mutants. To verify that secondary mutations outside the gB gene had not been introduced into KOS DNA during cotransfection, pKBXX was used to rescue mutations in K082 and KΔT. For both viruses, the marker rescue frequency was 5%, compared with <0.02% for control transfections in which pKBXX was not included. These data indicate that the defects in K082 and KΔT are fully explained by mutations within the 4-kb gB sequence.

Expression of the mutant virus proteins. To determine whether virus mutants could express the expected gB polypeptides, extracts of infected HEL cells were electrophoresed on polyacrylamide gels and subjected to Western hybridization. In preliminary experiments a gB-specific polypeptide for KΔT was observed that was approximately 35 to 40 kilodaltons smaller than that of KOS (Fig. 4; data not shown). When D6 cells were used, a polypeptide was observed at the same mobility as on HEL cells, as well as the mature gB band expressed from the chromosomal copy of gB. Chromosomally expressed gB was detectable only after superinfection. HEL cells were used in labeling experiments because of the known protease degradation in Vero cell extracts (30). No polypeptide was detected for K082 infections. Therefore, in the following experiments only KΔT virus was used.

The membrane-spanning region has been deleted in KΔT,

FIG. 3. Genomic structure of the gB mutant viruses. Small batches of viral DNA were digested with the restriction enzymes indicated and subjected to Southern blot hybridization as described in Materials and Methods and in the legend to Fig. 1. The lower diagram is a restriction map for the indicated enzymes used in the Southern blot. Abbreviations: B, *Bam*HI; H, *Hind*III; Hp, *Hpa*I; T, *Bst*EII. The numbers represent the sizes of the relevant restriction fragments in kilobase pairs.

FIG. 4. Processing and secretion of the gB-specific polypeptide in K Δ T. Monolayers of HEL cells in 60-mm-diameter petri dishes were infected with KOS or K Δ T virus at an MOI of 10 PFU/cell and incubated at 37°C. Six hours after infection the cultures were pulse-labeled with 100 μ Ci of [3 H]mannose in 0.4 ml of TBS at 37°C for 20 min. Pulse-labeled cell extracts (lanes pe) were prepared in lysis buffer from some of the dishes. The other cultures were further incubated in medium and chased for an additional 2.5 h, and then both cell extracts (lanes ce) and extracellular medium (lanes cm) were collected. All these samples were precipitated with a pool of monoclonal anti-gB antibodies (B3, B4, B6, and B9). The protein samples were separated by electrophoresis on 9% SDS-polyacrylamide gels.

which may result in secretion of the gB polypeptide. Alternatively, the shortened polypeptide may not be glycosylated, processed, and transported to the cell surface. To investigate these possibilities, pulse-labeled ([3 H]mannose) and pulse-labeled and chased infection cells and growth medium were treated with a pool of four gB-specific monoclonal antibodies (Fig. 4). For KOS infections, gB precursor pgB was synthesized during a 20-min pulse-labeling beginning at 6 h postinfection, and much of it was converted to mature gB during a 2.5-h chase interval. Although no gB

FIG. 5. Immunoprecipitation of the K Δ T shortened gB polypeptide by individual monoclonal antibodies. Monolayers of HEL cells in 60-mm-diameter petri dishes were infected at an MOI of 10 PFU/cell and incubated at 37°C. The cultures were labeled with 20 μ Ci of [35 S]methionine in 1 ml of medium from 6 to 20 h postinfection. Cell extracts and extracellular medium were prepared and immunoprecipitated with individual anti-gB antibodies (B3, B4, B6, and B9). The samples were electrophoresed on 9% SDS-polyacrylamide gels. Lanes 1, KOS cell extract; lanes 2, K Δ T extracellular medium.

appeared to be secreted into the extracellular medium, longer exposures to X-ray film showed a faint band in this lane. The shortened gB polypeptide precursor specified by K Δ T was processed to the mature form more quickly than KOS-specified gB. Some molecules were processed during the pulse-labeling interval. After 2.5 h most of the shortened polypeptide molecules were processed, and about half of them were secreted into the extracellular medium (compare the last two lanes of Fig. 4).

FIG. 6. Polypeptide and glycopeptide composition of mutant viral particles. HEL cells (10^8) in roller bottles were infected at an MOI of 10 PFU/cell and incubated at 34°C. The infected cells were labeled with 150 μ Ci of [14 C]glucosamine or [35 S]methionine in 10 ml of medium from 6 to 24 h postinfection. Viral particles were purified by dextran T10 gradient centrifugation. Proteins were analyzed in 9% SDS-polyacrylamide gels.

TABLE 2. Recombination between the HSV-1 sequences in D6 cells and in mutant viruses^a

Stock no.	Wild-type frequency	
	KAT	K082
1	3.3×10^{-6}	1.3×10^{-6}
2	$<7.1 \times 10^{-8}$	5.6×10^{-8}
3	3.4×10^{-5}	1.9×10^{-5}
4	4.1×10^{-7}	$<4.9 \times 10^{-6}$
5	$<1.1 \times 10^{-6}$	$<2.5 \times 10^{-6}$
6	$<7.8 \times 10^{-7}$	$<5.9 \times 10^{-6}$

^a Stocks 1, 2, 4, 5, and 6 were prepared from roller bottle cultures of D6 cells infected with the contents of a 25-cm² flask of D6 cells previously infected with a single plaque. Stock no. 3 was prepared from D6 cells in a roller bottle infected at an MOI of 0.01 PFU/ml from a primary stock (27). Progeny viruses were quantitated on D6 and Vero cells. Wild-type frequency was calculated as the ratio of the titer on Vero to that on D6 cells.

To determine whether the shortened polypeptide of KAT had lost some of its antigenic determinants, KOS-infected cell extracts and growth medium from KAT-infected cells (labeled from 6 to 20 h postinfection with [³⁵S]methionine) were treated individually with the same four monoclonal antibodies. All of the antibodies, designated B3, B4, B6, and B9 (25), precipitated a KOS product, and B3, B4, and B6, but not B9, precipitated the KAT shortened polypeptide (Fig. 5).

Formation of mutant viral particles. The experiments in this section were initiated to determine whether gB is required for viral particle assembly, especially for nucleocapsid envelopment. Infected HEL cells, doubly labeled with [³H]thymidine and [³⁵S]methionine, were purified by dextran T10 gradient centrifugation, and peak fractions were further purified by sucrose gradient centrifugation. A major peak containing both ³H and ³⁵S was detected for K082 and KAT in the same position in the sucrose gradient as for KOS (data not shown). This suggests that the mutant viruses could form particles of the same density and rate of sedimentation as the wild type.

To compare the polypeptide and glycopeptide profiles of mutant viral particles with those from KOS, the peak fractions from dextran T10 gradient centrifugation were combined and subjected to SDS-polyacrylamide gel electrophoresis. All the polypeptides labeled by [³⁵S]methionine except gB were present in the particles of K082 and KAT (Fig. 6). The polypeptide with slightly slower mobility than gB is probably VP7.5. Furthermore, all the glycoproteins except gB were present when [¹⁴C]glucosamine labeling was used. The same amount of sample was loaded onto the gel for KOS, K082, and KAT and produced a similar signal. Together the data indicate that the mutants could form a similar number of enveloped virions compared with KOS and that the virions contain all viral polypeptides and glycopeptides except gB.

Recombination between the sequences of the gB gene. KAT, K082, and other mutants that contain insertion or deletion mutations will not spontaneously revert to wild type. However, recombination will occur between the mutant viral genome and the HSV-1 sequences inserted into the cellular chromosome of D6. Therefore, stocks were grown on D6 cells and the resulting viruses were tested for wild-type recombinants (the ratio of the number of plaques formed on Vero relative to those formed on D6 cells) (Table 2). The frequency was generally low and ranged from $<7.1 \times 10^{-8}$ to 3.4×10^{-5} for KAT and from 5.6×10^{-8} to 1.9×10^{-5} for K082. Nonreproducibility implies that this is a rare event.

Recombination may sometimes occur early in the development of a particular stock and result in the higher frequencies observed. Nevertheless, most of the stocks, especially those prepared from single plaques, were relatively free of recombinant viruses. If cells were transformed with pK082 and infected with virus mutants such as KAT, the number of plaques formed would be a measure of the number of recombination events. By using a set of mutations with different deletion endpoints, the homologous recombination frequency could be measured as a function of the number of nucleotides between the mutation in the transformed cells (pK082) and the one in the infecting virus.

The frequency of wild-type recombinants that occurred between the mutated gB genes of KAT and K082 was also determined by coinfection of D6 cells with both mutants (Table 3). The resulting progeny virus that could form plaques on Vero cells were scored as recombinants. The frequency was reproducible and averaged 7×10^{-3} , or 0.7%. Compared with that between D6 and the mutant viruses, this frequency is very high. The distance between the mutations of KAT and K082, where crossover occurs, is 2.1 kb.

DISCUSSION

A cell line transformed with an essential gene of HSV-1 can be very useful as a host for the isolation and propagation of mutant viruses defective in that gene, as successfully demonstrated by DeLuca et al. (11). Expression of an essential gene in a transformed cell allows the transfer of any type of mutation (missense, nonsense, frameshift, insertion, or deletion) that can be constructed in cloned genes in vitro to viral DNA. Mutations that are normally lethal are conditionally lethal in the transformed cell, so that mutant virus stocks can be prepared for study in the lethal condition (in an untransformed or nonpermissive host). The present study is the first one to use transformed cells to isolate and propagate viruses whose mutations specify a structural protein of the virus. This cell line should prove useful for the study of gB functions in glycoprotein processing and transport, intermolecular interactions, viral assembly, viral entry, and virus-induced cell fusion.

Another possible use for the type of virus mutant described here is to map antigenic determinants expressed by gB and recognized by gB-specific monoclonal antibodies. The four monoclonal antibodies used include representative of epitope groups I (B3), II (B9), III (B4), and IV (B6) (25). All of the antibodies precipitated gB from KOS infections, and only B9 failed to precipitate the secreted gB polypeptide expressed in KAT infections. B9 is a neutralizing antibody, implying that it recognizes a site of gB exposed outside the membrane. Therefore, the antigenic determinant for B9 may

TABLE 3. Recombination between KAT and K082^a

Virus seeded	Growth (PFU/ml)		Wild-type frequency
	D6	Vero	
KAT	4.6×10^6	$<5 \times 10^0$	$<1.1 \times 10^{-6}$
K082	3.1×10^7	$<5 \times 10^2$	$<1.6 \times 10^{-5}$
KAT + K082	2.2×10^7	1.4×10^5	0.065
KAT + K082	1.5×10^7	6.5×10^4	0.045
KAT + K082	1.2×10^7	1.2×10^5	0.01

^a D6 cells (approximately 2×10^6) in 60-mm-diameter petri dishes were infected with KAT or K082 at an MOI of 10 PFU/cell or with both viruses (KAT plus K082) at an MOI of 5 PFU of each per cell and incubated in 2 ml of growth medium at 37°C for 24 h. Progeny viruses were quantitated on D6 and Vero cells. Wild-type recombinants were calculated as the ratio of the titer on Vero to that on D6 cells.

be located between amino acids between residues 552 and 727 (the first residue of the membrane-spanning domain). Alternatively, the B9 recognition site may not be confined to the deleted residues, and its recognition site may have been altered by the KΔT deleted residues.

KΔT contains a 323-amino-acid deletion, prompting us to ask whether this alteration affects processing and transport. Since the membrane-spanning domain of gB in KΔT is deleted, the polypeptide would be secreted rather than incorporated into the plasma membrane if transport were not blocked by the mutation. Our results indicate that this polypeptide was efficiently processed and transported. Within 20 min, about half of the polypeptide precursor had been converted into high-molecular-weight forms, and the majority of the polypeptide was processed in 2.5 h, implying that transfer from the rough endoplasmic reticulum to the Golgi apparatus is rapid. Approximately one-half of the shortened polypeptide was also secreted in the same time interval. Interestingly, molecules secreted extracellularly were heterogeneous and larger in apparent molecular weight than the intracellular molecules. Heterogeneity may be related to increased polypeptide glycosylation caused by the deletion mutation.

In contrast to our observations on the shortened polypeptide of KΔT, a 12-nucleotide deletion in the cysteine-rich region of low-density lipoprotein (LDL) receptor in Watanabe heritable hyperlipidemic rabbits reduces the rate of transport of this protein to the cell surface (45). This mutation blocks the processing of LDL receptor to the mature form, apparently through inefficiency in its transport from the rough endoplasmic reticulum to relevant parts of the Golgi apparatus (14). All the gB *ts* mutants of HSV-1 that have been characterized produce gB precursor but not mature gB at the nonpermissive temperature, even when chased at the permissive temperature (12). These mutants, like the LDL receptor mutation, are not processed. It will be interesting to determine whether some secretory mutants are also blocked in processing, unlike the findings for KΔT polypeptide. Perhaps functional protein domains are a requirement for processing and transport.

The requirements for envelopment of HSV-1 nucleocapsids are largely unknown. In other enveloped animal viruses, like rhabdoviruses, paramyxoviruses, and orthomyxoviruses, there is between the envelope and the nucleocapsid a protein designated M which may play a role in recognition between proteins in the two structures (see, for example, reference 8). For HSV-1 no M protein has been identified, but it is possible that the cytoplasmic domains of viral glycoproteins are responsible for this interaction. Among the HSV-1 glycoproteins with known DNA sequence data, only gB and gE have a long cytoplasmic sequence that could form a functional domain (6, 26). The gE gene was recently identified as an unessential gene since deletion in this gene does not affect viral replication (23). Therefore, gB as a major envelope protein is the best candidate to interact with the nucleocapsid. The gB-defective mutants are unique in determining the role of gB in viral envelopment and assembly because they do not synthesize gB as a transmembrane protein. In KΔT, the shortened peptide becomes secretory. K082 produces the first 43 amino acids of gB, including the signal sequence. This polypeptide is not long enough to extend outside cytoplasmic ribosomes to interact with the signal recognition particle and pass through the rough endoplasmic reticular membrane (43). It is probably degraded in the cellular cytoplasm. Nevertheless, these two mutants were able to form virus particles with the same polypeptide

composition and the same envelope polypeptide composition as KOS, except for the absence of gB. It is concluded that gB is not essential for nucleocapsid envelopment or viral assembly.

In summary, we have described a system for studying the role of gB in HSV-1 adsorption, entry, and cell fusion. A gB-transformed Vero cell line (D6) has been established which is permissive for gB mutant growth and thus is useful for isolation and preparation of mutant viral stocks. Structural or functional changes in such mutant stocks may then be ascertained through transfection experiments with nonpermissive cell lines. The potential of this system has been demonstrated through use of K082 and KΔT, two HSV-1 mutants defective in the gB gene. More such mutants are needed to continue these studies, and for this purpose a large number of *Hpa*I linker insertion mutants, as well as a small number of deletion mutants isolated by recloning pairs of *Hpa*I insertions, have been developed. Their characterization should ultimately lead to an understanding of the functional correlates of particular gB-coding regions.

ACKNOWLEDGMENTS

We acknowledge discussions of data with our colleagues Chitrita DebRoy, Patricia MacKeen, and Nels Pederson. Dr. MacKeen isolated the plasmid pKΔT for these studies.

This research was supported by grant MV-167B from the American Cancer Society.

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